The species problem in protozoa revisited

The biological species concept as coined by Ernst Mayr is not applicable to many protists which reproduce by inbreeding or asexually. An extended concept supplementing the biological species concept was suggested by T. M. Sonneborn after intensive studies on differently reproducing species of the Paramecium aurelia complex. In his concept based on the hypothesis that inbreeding or asexually reproducing taxa also evolve as discrete units, he suggested that a species should be recognized as an evolving entity that has undergone a threshold of minimum evolutionary divergence. However, Sonneborn's idea was poorly received. We examine different morphological and molecular characters discovered and applied in taxonomy since Sonneborn developed his hypothesis. We conclude that there is now an abundance of objective characters to arrive at sound judgement about the complexity of the genetic differences necessary to delimit species in Sonneborn's sense when the biological species concept is not applicable. In addition, combined morphological and molecular studies reveal that, although many free-living protists may be globally distributed, geographical patterns and local distribution also occur.

Key words: Asexual reproduction; Evolutionary threshold; Inbreeding; Protozoa; Species concept.

1. The species problem in protozoa

The word "biodiversity" has spread more rapidly through the scientific community and popular culture in the last few years than probably any term ever has in the history of science (O'Hara 1994). However, we still have problems to understand what the systematic basis of biodiversity, a species, is. It is often difficult to understand whether two populations that differ slightly from one another (and may be geographically separate) are two different species or varieties of the same species. This species problem has been realised by systematists since the early 19th century and a large group of species concepts has been developed since then. Furthermore, we are viewing an accelerating debate on the question whether free-living protists are local or global in their distribution (Foissner 1999; Finlay 2002), which, in our opinion, is also tightly linked to our understanding of what a species is.

An outstanding and widely accepted theory promoted by Ernst Mayr (Mayr 1963, 1969) is the biological species concept. The great advantage of this concept is its objective genetic criterion of fertile reproduction within a gene pool and its isolation from other gene pools. Unfortunately, and as is well known, this "truth" is not applicable to inbreeding and asexually reproducing organisms which however evolved as successfully as sexually outcrossing species. A solution or circumvention of this problem has not been achieved to date, and as biological historians point out, was even prevented by mighty proponents of the biological species concept (Schloegel 1999).

An unexpected drama happened at the annual meeting of the American Society for the Advancement of Science in Atlanta in December 1955 at a symposium organized by Ernst Mayr on the species problem. The symposium contributors repeatedly endorsed the biological species concept and minimized its problematic aspects - until protozoan geneticist Tracy M. Sonneborn took his turn at the podium (Schloegel 1999). During the lectures he had grown frustrated, not by the positive claims of the biological species concept, but rather by what the speakers radically excluded, namely all obligatory inbreeding and asexual organisms with which he was familiar after many years of intensive study of a wide range of protozoa, including numerous species of

the Paramecium aurelia complex. He consequently discarded his prepared manuscript and attempted spontaneously to address the problem more comprehensively. This attempt was developed further and the results were published in the symposium volume (Sonneborn 1957).

Sonneborn was far from rejecting the biological species concept, but rather attempted to expand its theoretical relevance in the light of his genetic studies on Paramecium. He argued for a broader species concept which would embrace the whole of biology, since "... on this (biological) concept, perhaps half or more of the species recognized by students of protozoa are invalid and, in principle, cannot be modified so as to become valid. In like manner, practically every phylum of invertebrates and all major plant groups contain numerous organisms in which species do not exist at all or in which each individual is a separate species. This greatly limits the applicability of the modern species concepts" (Sonneborn 1957). He basically assumed that most fundamental to an organism's evolutionary success was its ability to maximize reproduction and to maintain enough genetic variability to respond to the demands of a changing environment. Central to his conception of speciation in protozoa was the argument by Sewall Wright, that small populations of inbreeding and asexual organisms result in the fastest and most effective process of selection, shifting populations in adaptive peaks, separated from others through adaptive valleys, which had been attributed by Dobzhansky only to sexual species (Wright 1940; Sonneborn 1957). Thus, also asexually reproducing forms would build discrete evolving groups. Finally, Sonneborn came up with an enlarged species concept on the principle of minimal irreversible evolutionary divergence. Basically, he postulated that species in sexual and asexual organisms can be defined on essentially the same principle, which is the surpassing of a threshold of minimal irreversible evolutionary divergence (Sonneborn 1957).

Sonneborn suggested that means to ascertain such differences for asexual organisms were to be found in detailed comparative studies on life cycles, morphology, cytology, physiology, and ecology: "Every possible means of arriving at sound judgement about the complexity of the genetic differences and their discontinuities would have to be utilized. This would be a great labour". However, such labour, he pointed out, had been required for the successful analysis of sexual species, too (Sonneborn 1957).

The reception of Sonneborn's ideas was poor. Mayr argued that the breakdown of the biological species concept when applied to asexual organisms, "has led some authors to go one step farther and abandon the biological concept altogether". Thus he transformed Sonneborn's attempt to achieve to a more comprehensive species concept into one that rejected the biological species concept in its entity, which never had been Sonneborn's aim. Mayr argued in his revised concluding comments for the published congress volume (Mayr 1957), that "widespread though it is in certain groups of organisms, asexuality is an exception rather than the rule." (cited after Schloegel 1999). Similarly, G.G. Simpson judged Sonneborn's contribution as "a major work on some aspects of protozoology" (Simpson 1958). Thus it is not surprising that it attracted little attention as a contribution to evolutionary theory (Schloegel 1999). Astonishingly, Simpson himself formulated his evolutionary species concept later as a broader alternative to the biological species concept in order to relate genetic species to the evolutionary process and to omit the criterion of interbreeding (Simpson 1961), not unlike Sonneborn's suggestions.

2. The further development

Systematic research work in protozoa continued nevertheless. This is to an important part due to (i) the fact that several protists became important model organisms in molecular biology, (ii) that the pivotal role of protozoa in ecological food webs has been further understood in recent years and (iii) the remarkable endurance of outstanding protozoologists. The following section reviews some of the, in our view, important aspects of protist research that impact on the species

problem, recognising it is a far from complete list. This will allow a test of Sonneborn's hypothesis that, although maybe laborious, objective criteria can be found to identify evolutionary entities that underwent significant genetic differentiation which may be addressed as thresholds of irreversible genetic change, i.e. to delimit species when the biological species concept may not be applicable.

In addition, we will consider the ongoing dispute which is tightly connected "with the species problem, namely the alternative hypotheses that (i) roughly every species is dispersed everywhere (Finlay and Fenchel 1999; Finlay 2002) or (ii) geographically restricted patterns also occur (Foissner 1999; Foissner et al. 2001).

2.1 Morphological analyses

Several research groups have maintained [alpha]-taxonomic research work and described new species on morphological characters of live specimens and for example after protargol impregnation, occasionally with additional morphogenetic investigations (Liiftenegger et al. 1988; Foissner 1998; Lee and Patterson 1998; Al- Qassab et al. 2002). Also important work was the unravelling of taxonomic confusion caused by others (Foissner and Berger 1999). Although mainly addressing the morphological species concept, the differences they use to delimit species may also be interpreted as indicators for irreversible genetic differences that have evolved (an analogous conclusion that is also involved in the biological species concept, although many taxonomists may of\ten not be aware of it). Based on their findings, the authors vigorously vote for geographical patterning of protozoan species distribution, besides acknowledging that many species may also be globally distributed. Increasingly, those data are fruitfully combined and evaluated with other evidence, such as molecular data (Foissner et al. 2001), putting more evidence on threshold values achieved by the species under study.

2.2 Morphometric analyses

Another approach based on morphology was undertaken by Gates (1977). he studied morphometric variation of cirral patterns in ciliates claiming that (i) objective data based on repeatable measurements when (ii) treated with standard statistical treatment such as multivanate analyses are (iii) more sensitive and will yield higher resolution power to analyse population structure than for example allele frequencies. he came to the conclusion that a reasonable classification can be based on types of ventral cirral patterns and dorsal silverline systems (argyrome). In consequence, he suggested to lump species, e. g. the Euplotes vannus - crassus minuta group in one species E. vannus, albeit he acknowledged that other evidence for considerable genetic differentiation has been going on within such morphologically defined, phenotypic limits (Gates 1977). In our view, this gross morphospecies concept was of limited help to the species problem in Euplotes, whereas in other cases, such as Paramecium and Tetrahymena the morphometric approach could recover most of the species (Powelson et al. 1975; Gates and Berger 1976).

2.3 Molecular data

2.3.1 Isoenzyme electrophoretic patterns

Different kinds of molecular data were discovered to be informative for taxonomic and higher level systematics. One of the earliest was isozyme patterns after electrophoretic separation. Indeed, Tracy Sonneborn, who had refrained from giving latin species names to the fourteen

Paramecium aurelia varieties or syngens promptly did so after diagnostic isoenzyme patterns were discovered in the pioneering work of Andrew Tait (Tait 1970; Sonneborn 1975). Isoenzyme patterns were successfully applied to a wide range of protists (Bordon et al. 1973, 1977; Genermont et al. 1985; Schlegel 1985; Valbonesi et al. 1985, 1988; Schlegel et al. 1988). Cryptic or sibling species could be separated as "well as asexually reproducing lineages, for example in the cihate genus Tetrahymena. Quantitative estimates of the percentage of alleles that are shared by individuals of a species were made in this genus and it was suggested that similar values of allelic identity, namely around 67%, could be used to separate asexual lineages as species (Borden et al. 1973, 1977). Isoenzyme patterns proved to be extremely stable. In a study on the genus Stylonychia, Ammermann and his coworkers compared isoenzyme patterns of strains from distant areas such as North America, Europe and the People's Republic of China (Ammerman et al. 1989). They discovered high genetic identity within the species S. lemnae and 5. mytilns, and diagnostic enzyme bands for each species. In summary, these are good arguments that isoenzyme patterns are of diagnostic value to discern species, including those that are reproducing asexually. With regard to the local versus global dispute of species distribution, isoenzmye patterns have proved that there are widely distributed protists which are still able to interbreed successfully, as demonstrated by heterozygous enzyme patterns in offspring clones (Ammermann et al. 1989).

2.3.2 DNA fragment analyses

More sophisticated techniques were developed, based on the enzymatic amplification of DNA. This approach largely overcomes the drawback of the requirement for large amounts of cells needed for enzyme electrophoresis. Species separation was successful, e.g. in different genera of ciliates, amoebae, and flagellates, by comparison of restriction enzyme patterns of PCR amplified ribosomal DNA after electrophoretic separation (Jerome and Lynn 1996; Clark and Diamond 1997; Lin Lim et al. 2001; Chen and Song 2002; Shang et al. 2002; Chen et al. 2003). These markers are, as well as Random Amplified Polymorphic DNA, in our view, sound characters that may contribute to a clear judgement about the complexity of the genetic differences and their discontinuities between species. Interestingly, a phylogenetic analysis of Euplotes species (Shang et al. 2002) came up "with a tree completely compatible with one that had been published 14 years before based on enzyme electrophoresis (Schlegel et al. 1988), emphasizing the soundness of the characters under study.

The sensitivity of RAPD analyses was impressively demonstrated by population studies using even single cells for PCR-amplification (Kusch and Heckmann 1996). Besides the determination of intraspecific diversity and interspecific divergence of three Euplotes species, a clonal population structure with low genetic diversity of only two different genotypes was discovered in E. daidaleus. Thus, with respect to the species problem, it may be important to emphasize that also sexually reproducing organisms may have a more or less clonal population structure, which has often been proposed, but for which Kusch and Heckmann provided convincing genetic evidence. Similarly, four genotypes were detected in natural populations of the ciliate Stentor by Kusch (1998), in a comprehensive RAPD analysis of more than 700 individuals, over a geographic range of 500 km. Remarkably, those genotypes were not randomly distributed, but, on the other hand, no spatial correlation could be observed, i.e. closer populations were not more genetically similar to one another.

2.3.3 Comparison of gene sequences

Gene sequence comparisons have been increasingly and predominantly applied to reconstruct phylogenetic relationships. However, such analyses are also informative at the species level and have even led to the discovery of many new protistan taxa, often in extreme habitats, for example in the Rio Tinto in Spain with a pH of 2. These results challenge traditional ideas about the phylogenetic range of organisms that are capable of living in extreme habitats (Amaral Zettler et al. 2002). Likewise freshwater monothalamous foramimfera have been detected with gene probes. Thus, foraminifera are by no means exclusively marine organisms (Holzmann et al. 2003). So called "picoeukaryotes" were also discovered in the Antarctic marine polar front; rRNA sequence comparisons place them in two lineages within the alveolates, related to the dinoflagellates, and with a sequence diversity equivalent to that displayed by all dinoflagellates (Lopez-Garcia et al. 2001). However, there has been a drawback in this approach until now. We only know the rRNA sequence of these organisms and nothing else; thus, important morphological, physiological and genetic data are missing. In addition, deep phylogenetic relationships reconstructed with rRNA have to be interpreted with caution (Philippe et al. 2000). An elegant approach to overcome this problem has been made recently by Massana et al. (2002), and was further developed by Stoeck et al. (2003). They used gene-specific rRNA oligonucleotides for fluorescence in situ hybridization to detect the cells in "water samples where the rRNA gene had been isolated; then they cut out the region containing the target cell and prepared it for scanning electron microscopy. Thus at least the gap to morphology has now been bridged.

In the following, we will present the results of combined morphological and molecular analyses in two examples, namely testate amoebae and foraminifera.

Testate amoebae have predominantly clonal population structures (Meisterfeld 2002a, 2002b). The classification is almost completely based on characters of the shell. Basic morphotypes of testate amoebae are very old. Precambrian (742 Mya) Paleoarcella athanata from the Chuar group in the Grand Canyon are strikingly similar to recent Arcella spp. (Porter et al. 2003). These ancient types are distributed worldwide. However, shell morphology is affected by environmental factors such as availability of building material, water content of the substrate, kind and availability of food (Wanner 1999). Thus, the question arises whether habitat- or region-specific morphs are modifications (ecophenotypes) or genetically distinct (species). Our phylogenetic analysis (Fig. 1) revealed that some broadly defined morphospecies are not monophyletic in the genus Englypha (Wylezich et al. 2002). A subsequent, more detailed morphological analysis then showed that genetically different isolates often have a distinct morphology, for example in shape and size of siliceous scales in the case of Euglypha, filifera (Fig. 2). Thus, in Sonneborn's concept these genetic differences may justify giving them species status. With respect to geographical distribution, what looks like support for the "every species everywhere" concept, might turn out to be a more complex and differentiated geographical patterning.

Fig. 2. A detailed morphological analysis shows that E. filifera isolates from Australia (left) and Costa Rica (right) also differ in morphology, namely in shape and size of siliceous scales (see arrow). Bars correspond to 20 m (upper row) and 10m (lower row).

Fig. 1. Maximum Likelihood tree based on small subunit ribosomal RNA. Euglypha fdifera isolates from Australia and from Costa Rica are not related and group in different clusters. Numbers at nodes are bootstrap pecentages.

This appears to be the case also in Globorotalia truncatulinoides, This extant plankton foraminiferan originated ~2.8 My ago in subtropical areas of the South Pacific, spread to all subtropical and temperate regions of the world's oceans, and expanded its range to southern subarctic waters. This wide geographic distribution is associated with a latitudinal morphological variability which was considered as ecophenotypic variation with\in one species. Analysis of rRNA revealed that there are four genetically distinct lineages which may be regarded as

different species. Species 1 and 2 characterize subtropical waters, species 3 is abundant exclusively in the Subantarctic Convergence, while species 4 inhabits subantarctic waters (de Vargas et al. 2001). These findings again are in favour of a more complex and patterned distribution of protozoa.

3. Conclusion

In summary, we tried to show that there are abundant objective characters to arrive at sound judgement about the complexity of the genetic differences necessary to delimit species in Sonneborn's sense. Therefore, we wish to make a plea to rethink his suggestions, emphasizing that they are supplementary to the biological species concept, and consider them in the future definition of the term species and in the practical taxonomic work of species delimitation (two aspects which should not be mixed up). Besides the proximal aspects of genetic isolation and evolutionary threshold, a comprehensive definition of the term species may also encompass the ultimate (ecological) and historical aspect as discussed by Sudhaus and Rehfeld (1992).

With respect to the competing hypotheses of global or local distribution of free-living protists we argue for a moderate view. Thus, while we can agree with Finlay and Fenchel that a large proportion of (particularly small) protist morphospecies are cosmopolitan, we also agree with Foissner that geographic patterns and local distribution of species occur as well. We argue for caution that we do not again, as in the case of Tracy Sonneborn, sacrifice the (whole) truth in favour of an appealing theory.

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